Detection of Low-Level Tumor Cells in Allergic Contact Dermatitis Induced by Mechlorethamine in Patients with Mycosis Fungoides

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Two patients with histologically proven mycosis fungoides, a malignancy of phenotypically mature T cells, received a topical challenge with mechlorethamine to areas of clinically uninvolved skin to exclude possible hypersensitivity reactions to this chemotherapeutic agent. In both patients, allergic contact dermatitis (ACD) developed at the sites of the application and resolved completely after withdrawal of mechlorethamine. The lesions were biopsied and analyzed for the presence of clonal T-cell receptor (TCR)-γ gene rearrangements using two polymerase chain reaction (PCR)-based assays involving denaturing gradient gel electrophoresis (PCR/DGGE) and ribonuclease protection analysis (PCR/RPA). The former method has a clonal detection threshold of 10^{-3}–10^{-2}, while the latter has a sensitivity of 10^{-5}. In both cases, the ACD lesions were polyclonal by PCR/DGGE. In contrast, PCR/RPA detected tumor-specific TCR-γ gene rearrangements in these same lesions. This indicates that the ACD lesions contained tumor cells at a density within the 10^{-5}–10^{-2} range. Analysis of peripheral blood mononuclear cells from both patients failed to detect the malignant clone and showed the same result as blood from four normal individuals. The normal skin from one patient also lacked detectable TCR-γ gene rearrangements. These results indicate that mycosis fungoides tumor cells are present within ACD lesions induced in mycosis fungoides patients and that this phenomenon does not appear to be due to the ubiquitous presence of detectable levels of these tumor cells in the blood or skin. These findings might be explained by nonspecific recruitment of malignant T cells to sites of local inflammation mediated by non-neoplastic antigen-specific T cells. Alternatively, they might be due to the local proliferation of very rare tumor cells in apparently normal skin in response to cytokines generated during the ACD reaction. In either case, the present study offers evidence that the malignant cells in mycosis fungoides retain at least some capability of responding *in vivo* to physiologic stimuli. ***Key words: mycosis fungoides/allergic contact dermatitis/T-cell receptor/PCR. J Invest Dermatol 106:685–688, 1996***

Mycosis fungoides (MF) is characterized by a clonal expansion of phenotypically mature T cells belonging to the skin-associated lymphoid tissue (SALT) (Bos, 1990). The typical phenotype is that of a $CD4^+CD45RO^+CLA^+$ memory T cell (Wood, 1992; Heald *et al*, 1993). The malignant clone of MF generally contains uniform rearrangements of T-cell receptor (TCR)-γ genes (Wood *et al*, 1994). The individual rearrangements vary not only by the usage of different variable (V) and joining (J) gene segments, but by virtually unique sequences of nucleotides at the junction of the participating segments—the combined result of small deletions of basepairs from the ends of the rearranging segments and the insertion of short stretches of random basepairs (so-called N region sequences) between the segments prior to joining. The individual TCR-γ gene rearrangement, including the sequence at the V/N/J junction, can therefore be regarded as a specific genotypic marker for the cells comprising the malignant clone in MF (Wood *et al*, 1994).

We have previously employed two sensitive molecular biologic methods to detect clonally rearranged TCR-γ genes in specimens obtained from MF patients. Both involve polymerase chain reaction (PCR)-based amplification of TCR-γ gene rearrangements followed by either denaturing gradient gel electrophoresis (the PCR/DGGE assay) (Wood *et al*, 1994) or ribonuclease protection analysis (the PCR/RPA assay) (Veelken *et al*, 1995). Both assays detect tumor-specific TCR-γ gene rearrangements based on their unique V/N/J sequences. PCR/DGGE has a sensitivity limit of 10^{-3}–10^{-2}, while PCR/RPA has a sensitivity limit of 10^{-5}. As a consequence, these two assays can provide complementary information regarding tumor clone density within MF patient samples.

In this report, we describe the use of these assays in two patients with MF to detect the presence of their malignant T-cell clones in lesions of allergic contact dermatitis (ACD) induced by mechlor-

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Abbreviations: MF, mycosis fungoides; ACD, allergic contact dermatitis; TCR, T-cell receptor; PCR/DGGE, polymerase chain reaction/denaturing gradient gel electrophoresis; PCR/RPA, polymerase chain reaction/ribonuclease protection analysis; PBMC, peripheral blood mononuclear cells.

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ethamine. Topical mechlorethamine is one of the major treatments for patch/plaque MF and its major side effect is allergic contact dermatitis (Haserick *et al*, 1959; Waldorf et al, 1967; VanScott and Kalmanson, 1973; Constantine *et al*, 1975; Price *et al*, 1977; Price *et al*, 1983; Vonderheid, 1984; Ramsay *et al*, 1988; Vonderheid et al, 1989). Our results indicate that MF tumor cells have the ability to participate in ACD reactions at low levels. This probably reflects their generally well-differentiated nature.

METHODS

Patients Patient 1 was a 43–year-old white female with newly diagnosed stage IB MF, although she reported her skin lesions began 20 years earlier. At the time of study, her MF lesions consisted of generalized poikilodermatous patches. Prior treatment included topical corticosteroids and ultraviolet B phototherapy. Patient 2 was a 73–year-old white female with a 19–year history of MF that was originally stage IIB. At the time of study, her MF lesions consisted of generalized thin plaques. Prior treatment included topical corticosteroids, localized electron beam therapy, localized orthovoltage radiation therapy and psoralen plus ultraviolet A phototherapy. Both patients were diagnosed using standard clinicopathologic criteria (Wood, 1992). Neither patient was undergoing treatment at the time of testing with 10 mg% mechlorethamine ointment applied nightly to a small area of uninvolved skin on one volar forearm. Within 1 wk, both patients developed allergic contact dermatitis manifested by pruritus, erythema edema, and mild vesiculation restricted to the site of mechlorethamine application. The test reaction subsided completely within 2–3 wk after discontinuing mechlorethamine. There was no appreciable change in either patient's MF lesions throughout this testing period.

Samples All biopsies were performed with informed consent and use of human subjects received committee approval. Peripheral venous blood and skin biopsies of the positive test site and distant MF lesions were obtained from both patients. In addition, a distant clinically normal skin biopsy was collected from Patient 1. Except for the lesional MF specimens from Patient 1, which were obtained about 1 month earlier, all specimens within each case were obtained concurrently 1 wk after beginning the nightly mechlorethamine use test. Normal peripheral venous blood was obtained from healthy volunteers. Mononuclear cells from blood specimens were purified by Ficoll density gradient centrifugation. All samples were cryopreserved at −70°C until further processing. DNA was extracted using routine procedures (Wood *et al*, 1994; Veelken *et al*, 1995).

PCR/DGGE Clonal TCR-γ gene rearrangements involving Vγ1–9 and Jγ1–2 were detected by two-round PCR using nested primer pairs followed by separation of PCR products in a denaturing gradient gel. The details of this method have been published previously (Wood *et al*, 1994). In this assay, ethidium bromide-stained gels exhibit a diffuse smear in lanes containing PCR products amplified from polyclonal T cells, while one or more distinct bands are present in lanes containing PCR products derived from T cells containing a dominant TCR-γ gene rearrangement. Furthermore, a matching band pattern in different lanes reflects an identical clonal TCR-γ gene rearrangement in the respective DNA samples. Titration studies using normal keratinocytes and tonsil cells have shown a sensitivity limit of 10^{-3}–10^{-2} for detection of a dominant T-cell clone (Wood *et al*, 1994).

PCR/RPA Tumor-specific TCR-γ rearrangements involving Vγ1–8 and Jγ1–2 were also detected using a second method known as PCR/RPA which has been described in detail previously (Veelken *et al*, 1995). An RNA probe for the clonal TCR-γ gene rearrangement was constructed by first amplifying DNA from the biopsy diagnostic for MF by PCR for 30 cycles using oligonucleotide primers complementary to conserved sequences in the Vγ1–8 and Jγ1–2 segments of the TCR-γ gene. The Jγ-specific primer carries at its 5′ end the promoter for T7 RNA polymerase. The incorporation of this promoter into the PCR product allows efficient *in vitro* transcription of the PCR product. Transcription in the presence of [α-^{32}P]UTP yielded a radiolabeled, anti-sense RNA probe specific for the malignant T-cell clone.

Unlabeled test RNA in sense orientation was synthesized in an analogous manner from the TCR-γ gene rearrangements found in the DNA of the ACD specimen, peripheral blood mononuclear cells (PBMC) from the patients, and PBMC from four healthy control donors. The normal skin specimen did not contain TCR-γ gene rearrangements amplifiable with the Vγ1–8 and Jγ1–2 primers. The Vγ-specific primer in this set of PCR reactions carries the T7 promoter. The Jγ-specific primer of this reaction binds to the Jγ segment in a position upstream of the primer used for synthesis of the probe. Test RNA and probe RNA were hybridized together under highly stringent conditions (85% formamide; 64°C). Mismatched pyrimidine residues in the resulting RNA heteroduplexes were then digested by RNase A at 42°C. The digested fragments were analyzed by polyacrylamide gel electrophoresis under denaturing conditions and subsequent autoradiography.

In this experimental design, only perfectly matching test RNA derived from the tumor-specific TCR-γ gene rearrangement protects the probe RNA from RNase digestion over the full length of the RNA heteroduplex. The presence of MF cells in the ACD specimen is therefore indicated by the detection of probe molecules corresponding in size to the full length of this duplex. The exact size of this fragment can be predicted from the size of the undigested probe and the relative positions of the primers used to synthesize the probe and test RNAs. Titration studies using normal bone marrow cells have shown a sensitivity limit of 10^{-5} for detection of the tumor clone-specific sequence (Veelken *et al*, 1995).

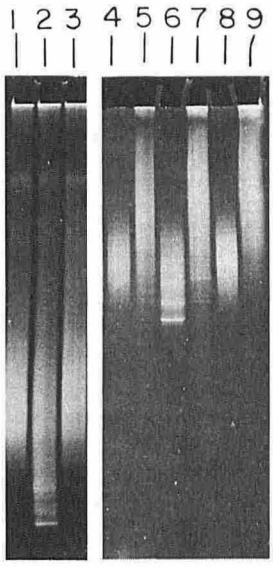

Figure 1. PCR/DGGE detects clonal TCR-γ gene rearrangements in MF lesions but not in ACD lesions induced in two MF patients. *Lanes 1–3*, Patient 1; *lanes 4–7*, Patient 2; *lanes 8* and *9*, polyclonal control. See text for details.

RESULTS

PCR/DGGE Detects Tumor Clone in MF but Not in Other Specimens Both patients showed clonal band patterns in their MF specimens by PCR/DGGE using Vγ1–8 primers. In addition, Patient 2 showed a Vγ9 clonal band pattern in her MF specimen. In contrast, there was no detectable clonal band pattern in the ACD or blood specimens obtained from either case. As in some normal skin biopsies lacking sufficient T cells bearing Vγ1–8/Jγ1–2 TCR-γ gene rearrangements, the normal skin specimen from Patient 1 showed no detectable TCR-γ PCR products by either agarose minigel analysis or DGGE. Representative PCR/DGGE results are illustrated in **Fig 1**. The first three lanes represent blood, MF, and ACD samples, respectively, from Patient 1. A distinct clonal band pattern is present only in the MF specimen (*lane 2*). *Lanes 4–7* represent samples from Patient 2. *Lanes 4* and *5* show a polyclonal pattern in the ACD sample analyzed for Vγ1–8 and Vγ9 rearrangements, respectively. *Lanes 6* and *7* show a dominant clonal pattern in the MF sample analyzed for Vγ1–8 and Vγ9 rearrangements, respectively. *Lanes 8* and *9* represent a negative control showing a polyclonal pattern for Vγ1–8 and Vγ9, respectively, in an unrelated case of cutaneous lymphoid hyperplasia.

PCR/RPA Detects Tumor Clone in MF and ACD but Not in Other Specimens Both patients showed a tumor-specific, rearranged TCR-γ gene sequence in their MF and ACD lesions by the PCR/RPA assay. In contrast, these was no detectable tumor-specific sequence in their blood specimens. As noted above, the normal skin specimen from Patient 1 lacked TCR-γ PCR products

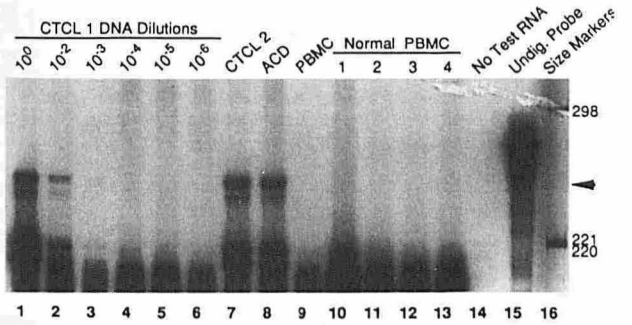

Figure 2. PCR/RPA detects identical clonal TCR-γ gene rearrangements in MF and ACD lesions obtained from Patient 1. See text for details. CTCL, cutaneous T-cell lymphoma; PBMC, peripheral blood mononuclear cells. Undig. probe, undigested RNA probe. The *arrowhead* points to the tumor-specific RNA fragment protected from digestion by the complementary tumor-specific RNA probe.

involving Vγ1–8 and Jγ1–2. Therefore, it was not subjected to clonal analysis by PCR/RPA.

Figure 2 demonstrates the results of the analysis of Patient 1. The probe (not subjected to hybridization and RNA digestion, *lane 15*) contains a strong clonal component corresponding to the tumor as well as a smear around the clonal band that presumably represents reactive lymphocytes frequently found in lesions of MF. When hybridized to test RNA synthesized from the same (*lane 1*) or a different (*lane 7*) MF lesion of this patient, a strong protected band in the expected position can be detected (*arrow*). A band of roughly similar intensity is present in the same position when test RNA from the ACD lesion is analyzed (*lane 8*). Absence of this band in the lanes representing four control PBMC (*lanes 10–13*) indicates the specificity of the protection from RNase digestion for the tumor-specific rearrangement. Absence of the same band in the lane of PBMC from the patient (*lane 9*) demonstrates that the signal in the ACD specimen is not merely due to a ubiquitous presence of the circulating malignant T-cell clone. Parallel analysis of logarithmic dilutions of DNA from the diagnostic specimen into normal PBMC DNA (*lanes 2–6*) allows an approximate comparison of the degree of involvement of the ACD lesion by cells of the malignant clone.

PCR/RPA applied to samples obtained from Patient 2 showed results similar to those of Patient 1 except that the concentration of tumor cells within the ACD lesion was lower. As shown in **Fig 3**, a fully protected band was visible in the lane corresponding to the ACD lesion (*lane 6*); however, its intensity was much reduced when compared to the diagnostic MF biopsy. The respective intensities of the bands of the ACD specimen and of the dilutions of DNA from the diagnostic MF sample (*lanes 1–5*) indicated that the density of malignant T cells present in the ACD was approximately 0.1% of

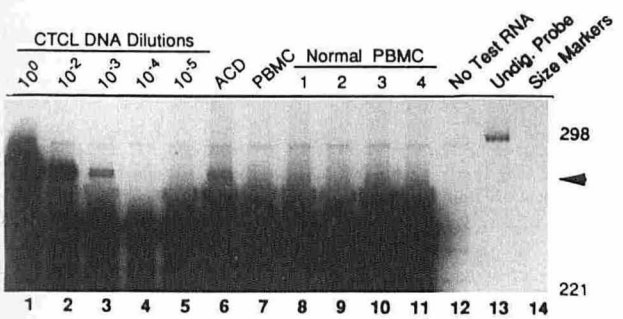

Figure 3. PCR/RPA detects identical clonal TCR-γ gene rearrangements in MF and ACD lesions obtained from Patient 2. See text for details. See **Fig 2** for abbreviations and explanation of the *arrowhead*.

that found in the MF lesion. The tumor-specific band was not detectable in PBMC from Patient 2 (*lane 7*) or in four control PBMC (*lanes 8–11*).

DISCUSSION

The results of the molecular biologic analyses performed in this study indicate that ACD lesions induced in MF patients contain low levels of the malignant T-cell clone detectable by PCR/RPA but not by PCR/DGGE. Based on the known sensitivity thresholds of these two assays, it is possible to estimate the MF tumor clone density in the ACD lesions to be within the 0.001–1% range.

In Patient 1, earlier Southern blot analysis of genomic DNA (Wood *et al*, 1994) had failed to detect clonal TCR gene rearrangements in the same MF patch lesion used to generate the PCR/RPA probe for the current study. This lack of detectable clonality by Southern blotting is not uncommon in early MF, because the tumor clone density is often below the 1–5% sensitivity limit of this technique (Wood *et al*, 1994). Given this information, the previously stated sensitivity limits of PCR/DGGE (10^{-3}–10^{-2}) and PCR/RPA (10^{-5}), and the observation that tumor clone densities in **Fig 2** were roughly similar for the MF and ACD lesions, we can estimate that the absolute density of tumor cells in both the MF and ACD lesions of Patient 1 lies within the 0.1–1% range.

Although Southern blotting was not performed on the MF lesion obtained for this study from Patient 2, prior quantitative studies indicated that thin plaques typical of her MF lesions contained approximately 25% T-cell DNA (Wood *et al*, 1991). If we assume that this is her maximum tumor cell density, then given the results of her PCR/DGGE and PCR/RPA analyses, which show about a 1,000–fold difference between the relative density of the tumor clone in the MF and ACD lesions (see **Fig 3**), we can estimate that the absolute tumor clone density within the ACD lesion lies within the 0.001–0.025% range. The lower density of tumor cells in the ACD lesion of Patient 2 relative to Patient 1 may reflect the longer duration of her MF and the cumulative effects of treatment on the functional capabilities of her tumor clone. Alternatively, it may reflect an inherent functional difference in her tumor. It is not due to a relative anergy to mechlorethamine, because the overall clinical extent of her ACD reaction was similar to that of Patient 1. It is tempting to speculate that allergens other than mechlorethamine might have induced a greater density of MF cells within the ACD lesions, because they would lack the chemotherapeutic effects of this allergen.

The absence of detectable tumor within the blood of both MF patients indicates that the presence of tumor cells within the ACD lesions was not due merely to the ubiquitous presence of low-level circulating tumor cells. Instead, it suggests that there had been selective trafficking of tumor cells into the skin of these patients at the site of ACD. This interpretation is also supported by the absence of detectable TCR-γ gene rearrangements involving Vγ1–8 and Jγ1–2 in the normal skin biopsy obtained from Patient 1. Although it is not possible to exclude the presence of occult tumor cells in other clinically normal skin locations, this absence of TCR-γ PCR products argues that the normal skin of Patient 1 was not a reservoir for occult MF that was incidentally detected during analysis of the ACD lesion. Alternatively, it is possible that very rare MF tumor cells were present in the normal skin and proliferated to a detectable level in response to cytokines released during the genesis of the ACD reaction.

Studies of ACD have shown that most of the lesional infiltrate is composed of T cells nonspecifically recruited to the site by a small vanguard of antigen-specific T cells. This vanguard is present at a maximum estimated density of 0.02–0.1% (Kalish and Johnson, 1990). Since each antigen generally contains multiple distinct epitopes, each clone of antigen-specific T cells is probably at a density of about 10–fold lower than this range (Mielke *et al*, 1994). In MF patients with ACD lesions, it is probable that the nonspecifically recruited, polyclonal T cells contain low levels of the MF tumor clone itself. This is not surprising given the well-differentiated $CD4^+CD45RO^+CLA^+$ SALT/memory T-cell phenotype of

most MF cases and the well known functional ability of MF tumor cells to traffic into the papillary dermis and epidermis—the same sites of T-cell infiltration seen in ACD.

Nevertheless, our findings should be regarded as being preliminary until additional cases can confirm them and perhaps extend them to other allergens. It will also be of interest to determine the tumor clone status of blood obtained at multiple time points and normal skin obtained at multiple sites during development of the ACD lesions. The clonality of the ACD sites at various times following their clinical resolution will also be important to determine.

Aside from providing new insights into the functional capabilities of MF tumor cells, the current study also complements prior studies of the molecular staging of MF patients using PCR/RPA (Veelken *et al*, 1995). These earlier studies showed detectable tumor cells in 100% of specimens including four morphologically uninvolved blood samples. The lack of detectable tumor in the blood specimens from the two MF patients in the current study indicates that tumor recognized by PCR/RPA is not ubiquitous in MF patients. If circulating tumor cells were present in these patients, the extent of blood involvement would have been below the 10^{-5} sensitivity threshold of PCR/RPA. This raises the possibility that molecular staging may provide prognostically significant information by determining the extent of tumor dissemination in MF patients more accurately than is possible using morphologic criteria.

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